의학박사 학위논문

Host Immune Response in *Staphylococcus aureus* Bacteremia: Differential Gene Expression of Toll-like Receptor 2 and Secretion of Cytokines

황색포도알균 균혈증 숙주 면역 인자 연구: Toll-like receptor 2의 발현과 사이토카인 분비

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The Department of Clinical Medical Sciences

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by
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A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Clinical Medical Sciences at Seoul National University College of Medicine

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이 논문을 의학박사 학위논문으로 제출함

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ABSTRACT

Introduction: *Staphylococcus aureus* is a commensal bacterium causing diverse infections from asymptomatic colonization to fatal infections. There have been many studies exploring host response to *S. aureus* infection. Toll like receptors (TLRs) play an important role in innate immune response against infection. Among the known TLRs, TLR2 is the main pattern recognition receptor recognizing pathogen-associated molecular patterns in *S. aureus* infection. The cytokines released by intracellular signaling pathways of TLR2 are involved in either beneficial or detrimental immune responses to the infection. We aimed to examine the relationship of TLR2 expression and the concentration of proinflammatory and anti-inflammatory cytokines with the prognosis in patients with *S. aureus* bacteremia (SAB).

Methods: Whole blood samples were collected at several time points categorized as within 5 days (≤ D5), between 6 to 9 days (D6-9), between 10-13 days (D10-13), between 14-19 days (D14-19) and after 20 days (≥ D20) of bacteremia, from patients diagnosed as SAB in Seoul National University Hospital and Seoul National University Bundang Hospital. We extracted RNA which was converted to cDNA by RT-PCR and separated plasma by centrifugation. The relative mRNA expression of TLR2 was measured by real-time PCR and the level of TNF-α, IL-6 and IL-10 was measured by Luminex mutli-bead assay. The findings were analyzed to identify associations with 30-

day mortality and severity.

Results: The level of TLR2 expression during the clinical course after the onset of bacteremia was variable among the 62 patients with SAB. In 10 patients with 30-day mortality, TLR2 expression was down-regulated and showed less dynamic changes throughout the whole period than in 52 survivors. IL-6 and IL-10 were significantly elevated in the mortal group. More patients (90%, 9/10) in the mortal group showed measurable IL-10 (>2.49 pg/mL) compared with the survived group (40%, 20/50) within 7 days of bacteremia. IL-6/IL-10 ratio tended to be either low or high in the patients with 30-day mortality, which implies excessive immune response of either proinflammation or anti-inflammation. The level of TLR2 expression within day 5 of bacteremia was higher in patients with severe clinical manifestations such as complicated bacteremia or septic shock.

Conclusions: In SAB patients, down-regulated TLR2 expression and elevated IL-6 and/or IL-10 at early stage of bacteremia were associated with 30-day mortality. TLR2 expression within 5 days of bacteremia was higher in patients with severe presentations implying hyper-immune response. Host immune response including TLR2 expression and secretion of cytokines may be a potential prognostic factor in SAB.

Keywords: *Staphylococcus aureus*, bacteremia, Toll-like receptor 2 (TLR2), cytokine

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Introduction

Staphylococcus aureus is a common human organism which acts in a variety way, from colonizer to pathogen of wide range of diseases. Due to its high rate of colonization and extensive spectrum of diseases it causes, many factors affecting its pathogenicity have been studied. The pathogenicity of *S. aureus* is due to the repertoire of toxins, exoenzymes, adhesins, and immune-modulating proteins that it produces (1). Increased antibiotic resistance and a high amount of virulence factors secreted by *S. aureus* contribute to its emergence as a pathogen (2).

Toll-like receptors (TLRs) are a central part of the innate immune response in bacterial infection (3). They are type I transmembrane proteins which consists of an extracellular domain and a cytoplasmic Toll/IL-1 receptor (TIR) domain and they act as pattern recognition receptors (PRRs) recognizing pathogen-associated molecular patterns (PAMPs). When ligands bind to TLRs, adaptor protein myeloid differentiation primary response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIPAP-inducing interferon (IFN)-β (TRIF) or TRIF-related adaptor molecule (TRAM) are recruited and signaling cascade is initiated involving IL-1R-associated kinase (IRAKs) and other kinases, which activates mitogen-activated kinases (MAPKs) and transcription factors such as nuclear factor κB (NFκB) and IFN-regulated factors (IRFs) (4). The final result of the activation of the cascade is increased expression and secretion of various AMPs, cytokines, and chemokines,

thereby recruiting immune cells to the site of infection and triggering of the adaptive immune response (5, 6). TLR2 in particular has been implicated in the innate immune response to *S. aureus* infection, recognizing both staphylococcal peptidoglycan and lipoteicholic acid (7).

The innate response to TLR2 ligation is primarily proinflammatory (8). In experiment with macrophages of mice, capturing of *S. aureus* via TLR2 induced proinflammatory reactions through activation of NFκB signaling pathways which was MyD88-independent (9). However, TLR2 signaling can also elicit anti-inflammatory response (10, 11). In a study using *S. aureus*, it is shown that differential TLR2 signaling leading to predominantly proinflammatory or modulatory responses is the result of selective involvement of the PI3K/Akt pathway in an APC-dependent manner (MΦs vs DCs) and differentially imprints the subsequent adaptive response as either an IL-10 response or a Th1/Th17 response (12). This divergent role of TLR2 in *S. aureus* infection has been widely studied showing various results and yet it is hard to clearly define the mechanism and function. TLR2 deficiency caused exaggerated proinflammatory response contributing to enhanced susceptibility (13) and decreased survival (7) in mouse model with *S. aurues* infection. This is supported by the studies in which TLR2 showed protective effects against *S. aureus* infections (14, 15). In contrast, absence of TLR2 was involved in protective effects in some models (16, 17). In mouse model, TLR2 deficiency prevented the down-regulation of CXCR2 and failure of neutrophil migration (16). Regarding genetically determined variation, no associations were found

between polymorphisms in the TLR2 genes and serious morbidity or mortality caused by *S. aureus* (18). However, there was an association between nasopharyngeal bacterial colonization and genetic variation of mannose-binding lectin (MBL), TLR2 and TLR4 in young infants in the study by Vuonovirta et al. (19). Stappers et al. also showed that polymorphisms in TLR1, TLR2, and TLR6 are associated with increased susceptibility to complicated skin and skin structure infections (20). Thus, it is still unclear whether TLR2 plays beneficial or detrimental roles in host immune response to staphylococcal infection, even in mouse models. It is suggested that a balance between proinflammatory and anti-inflammatory responses induced by TLR2 is a key factor of innate immune response in staphylococcal infections. Previous studies indicated that TLR2 activation induced by *S. aureus* leads to release of proinflammatory cytokines, mainly TNF-α (early) and IL-6 (late) (21, 22), and anti-inflammatory cytokine, IL-10 (10-12). When studied in mice, *S. aureus*-induced proinflammtory cytokine response was not dependent on macrophages and TLR2 deficiency resulted in decreased IL-10 release by macrophages, which contributed to dysregulated cytokine balance, impaired bacterial clearance, and mouse death (23). Most of these previous studies are based on in-vitro or in mice models, making it hard to apply in human patients with much more complex mechanisms and clinical heterogeneity. There are human studies of TLR2 in sepsis, and it is suggested that TLR2 expression is upregulated in septic patients and down-reglated in severe sepsis and septic shock patients, leading to death (24-26). Knowledge

about the regulation of TLR-induced signaling pathways may further elucidate the immune responses leading to favorable outcome and provide a new therapeutic strategy against severe sepsis (27). TLR4 and TLR2 are favorite targets for developing anti-sepsis drugs, and antagonistic compounds have shown efficient protection from septic shock in pre-clinical models (28).

The goal of this study was to determine the role of TLR2 signaling in *S. aureus* bacteremia (SAB) and its association with the clinical outcome. mRNA expression of TLR2 and level of proinflammatory and anti-inflammatory cytokines were measured at several time points during the course of bacteremia. We examined the changes in the level of TLR2 expression and cytokines at different phases of infection and how they differ according to prognosis.

Materials and Methods

Patients and samples

The patients over 18 years of age with SAB were enrolled in this study from March 2014 to April 2015 in 2 teaching-hospitals, Seoul National University Hospital, Seoul and Seoul National University Bundang Hospital, Gyeonggi-do. SAB was diagnosed if *S. aureus* was grown in 2 separate blood culture specimens or in 1 blood culture specimen if bacteremia was suspected clinically. Subjects with WBC count less than 4000/μl was excluded due to insufficient RNA.

Residual peripheral blood after routine CBC tests were collected in 5 different time periods: within 5 days (≤ D5), between 6 to 9 days (D6-9), between 10-13 days (D10-13), between 14-19 days (D14-19) and after 20 days (≥ D20) of bacteremia. Day 0 was defined as the day of the first blood culture positive for *S. aureus*. For those who discharged (transferred) or died within 14 days of admission, only 1 or 2 samples were obtained. If more than one sample is available within one period, the level of TLR2 expression was averaged between the 2 samples in that period.

Healthy volunteers were included in the study as a control group, who were free of inflammatory signs and underlying diseases.

This study was approved by the Committee of institutional ethics review board at Seoul National University Hospital and Seoul National University

Bundang Hospital. All subjects read and signed an informed consent (including healthy volunteers), unless considered as a waiver of informed consent.

RNA extraction and Reverse Transcription (RT)-PCR

RNA was extracted from whole blood in EDTA-containing tubes after plasma separation by centrifugation of 3000rpm for 15 minutes, using QIAamp RNA Blood Mini (Qiagen, Valencia, California) according to the manufacturer's instructions. Concentration and purity of RNA were measured by Nanodrop (Thermo Scientific, Delaware). Extracted RNA was either directly converted to cDNA by RT-PCR or freezed at -70°C.

RT-PCR was performed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, California). cDNA was kept at -70°C until performing real-time quantitative PCR (qPCR).

Quantitative Real-Time PCR (qPCR)

Real-time qPCR to measure TLR2 gene expression level was performed using TaqMan PCR. Commercialized kits were available for specific primers and TaqMan probes (Life Technologies, Carlsbad, California) for TLR2 and 2 housekeeping genes, GAPDH and β2-microglobulin. mRNA levels were quantified using an ABI7500 system (Applied Biosystems, Foster City, California). The expression of TLR2 mRNA was determined as the ratio of

TLR2 mRNA to GAPDH mRNA and/or β2-microglobulin mRNA, using $2^{-\Delta\Delta Ct}$ method (29, 30). Each sample was measured in triplicate and the mean value was used in the calculations. In order to decide the internal control gene, we have used both GAPDH and β2-microglobulin in some patients, and observed relatively similar patterns of TLR2 expression. The Ct value of β2-microglobulin tended to be below 20, therefore GAPDH was chosen as the internal control gene of this study. We used the MIQE guideline as a reference (31).

Quantification of plasma cytokine concentrations

Plasma was separated from whole blood and frozen at -70°C. The concentration of 3 cytokines, TNF-α, IL-6 and IL-10 was measured by Luminex technology using ProcartaPlex™ Human Immunoassays (affymetrix, eBioscience). Cytokine concentrations below the lower limit of detection were reported as the mid-point between the lowest concentrations measured and zero as described in other studies (32, 33).

Clinical outcomes

The primary clinical outcomes were 30-day all-cause mortality and complicated SAB. Complicated bacteremia was defined as one that resulted in persistent bacteremia (bacteremia present despite 7 days of appropriate therapy) or metastatic infections. Demographic data of the study subjects and

the following information on bacteremia were collected. (1) Early responsiveness defined as negative conversion of blood culture within 72 hours of appropriate treatment; (2) Onset of bacteremia classified into 3 groups: community-associated (CA) (bacteremia within 48 hours of admission without any healthcare associations within in 6 months), community-onset, healthcare-associated (CO HA) (bacteremia within 48 hours of admission with healthcare associations within in 6 months) and hospital-onset (HO) (bacteremia after 48 hours of admission); (3) Severity of bacteremia classified into 2 groups: Non-severe sepsis and severe sepsis including septic shock; (4) Methicillin sensitivity: Methicillin sensitive *S. aureus* (MSSA) and Methicillin resistant *S. aureus* (MRSA). These clinical features were examined for any associations with the expression of TLR2 and secretion of cytokines.

Statistical Analysis

The mRNA expression of TLR2 during bacteremia in each patient was plotted to compare the dynamics during the course of infection. The level of TLR2 expression and cytokine concentrations between groups were compared with a 2-sample t test or Mann-Whitney U test if the markers did not meet criteria for Gaussian distribution. Pearson correlation coefficients (r) of TLR2 expression and WBC with differential counts were calculated. The patients with unmeasurable and measurable level of cytokine were compared using

Fisher exact test. The correlation coefficients of cytokines were calculated with the Spearman correlation coefficient. *P*-values of <0.05 were considered statistically significant.

Results

Clinical and Microbiological characteristics

During the study period, a total of 290 patients with SAB were identified (Figure 1). Only 87 patients were assessed as eligible for this study, due to many reasons such as sample availability and WBC count. Among these patients, informed consent was obtained in 68 adult patients. Real-time PCR was failed in 6 patients leaving 62 patients for analysis. The clinical characteristics of the 62 patients are shown in Table 1. The age ranged from 19 to 85 years (mean 59.8 years, median 64.5 years) and 75.8% (47/62) was male. The most frequent source of bacteremia was from a secondary source and CRBSI was 24.2%. MRSA was isolated in 34 cases (54.8%).

Overall, the 30-day mortality rate was 16.1% (10/62), which was lower than previous Korean studies (34).

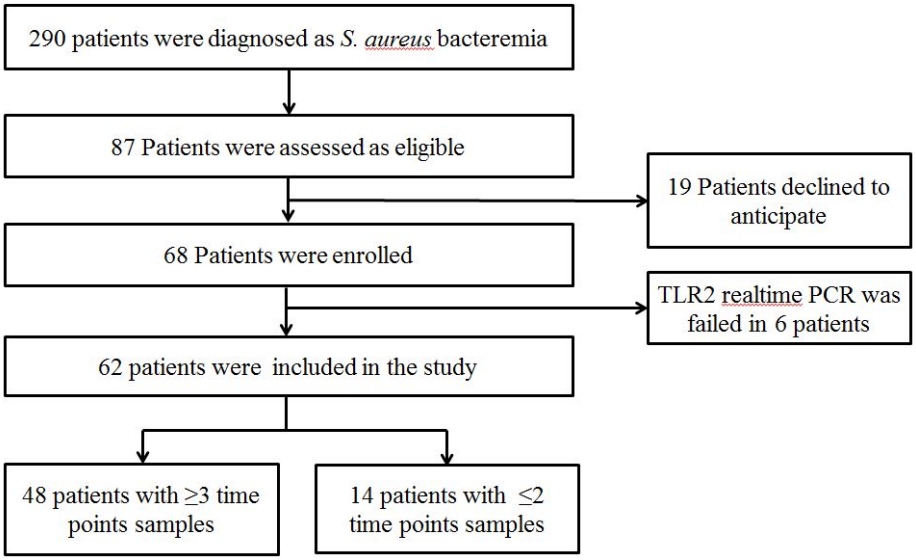


Figure 1. Enrollment of the patients.

Table 1. Clinical Characteristics of the Patients

Characteristics	N (%)[a]
Age (mean ± SD) (yr)	59.8 ± 17.0
Gender: Female (%)	15 (24.2)
Charlson's comorbidity weighted index (mean ± SD)	4.4 ± 3.1
No. of days total hospital stay (median [range])	28.0 (6-114)
Onset of infection	100 (73.0)
Community-associated	11 (17.7)
Community-onset, healthcare-associated	24 (38.7)
Hospital-onset	27 (43.5)
In ICU at first positive blood culture	31 (22.6)
Acute severity of illness	
Pitt bacteremia score (median [range])	1 (0-10)
SOFA score (mean ± SD)	5 ± 4.6
Severity	
Non-sepsis	10 (16.1)
sepsis	37 (59.7)
severe sepsis	8 (12.9)
septic shock	7 (11.3)
Site of infection	
Vascular catheter	15 (24.2)
Bone and joint	12 (19.4)
Skin and soft tissue	8 (12.9)
Unknown	6 (9.7)
Heart valve (native, artificial)	6 (9.7)
Respiratory tract	6 (9.7)
Implanted vascular device	5 (8.1)
Intraabdominal	2 (3.2)
Others	2 (3.2)
Treatment	
Inappropriate empirical	22 (35.5)
Inappropriate definitive	1 (1.6)
Time to administration of appropriate antibiotic (median [IQR]) (h)	23.9 (4.8-45.6)
30-day in-hospital mortality	10 (16.1)

[a] Otherwise specified

Changes in mRNA expression of TLR2 during the course of bacteremia

The samples were collected at around day 5, day 6-9, day 10-13, day 14-19 (range: day 2-48) after onset of bacteremia. The change in the level of TLR2 expression during the bacteremia was different among the patients, some showed increment while some showed decreasing tendency (Figure 2). The lowest level of TLR2 expression was 0.009 and the highest level was 6.482.

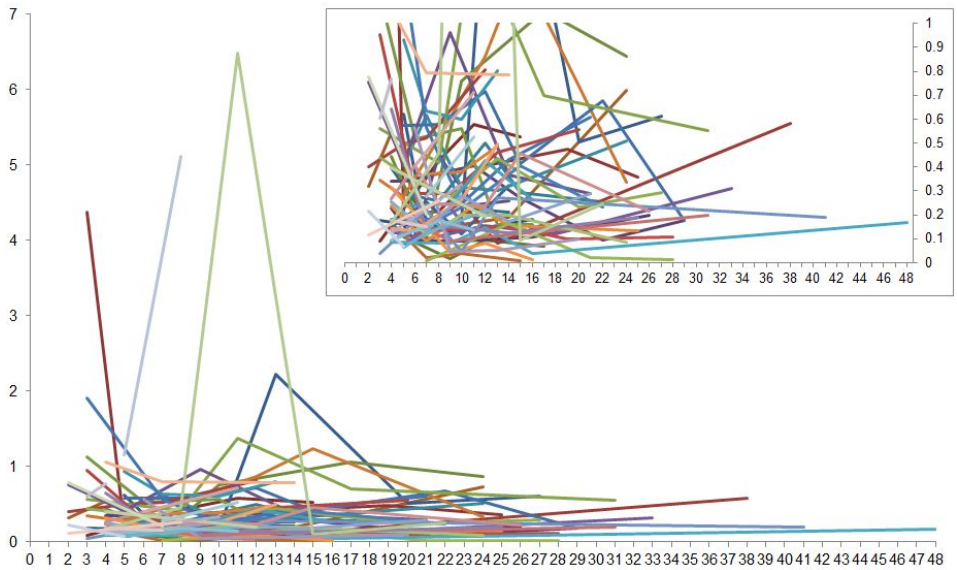
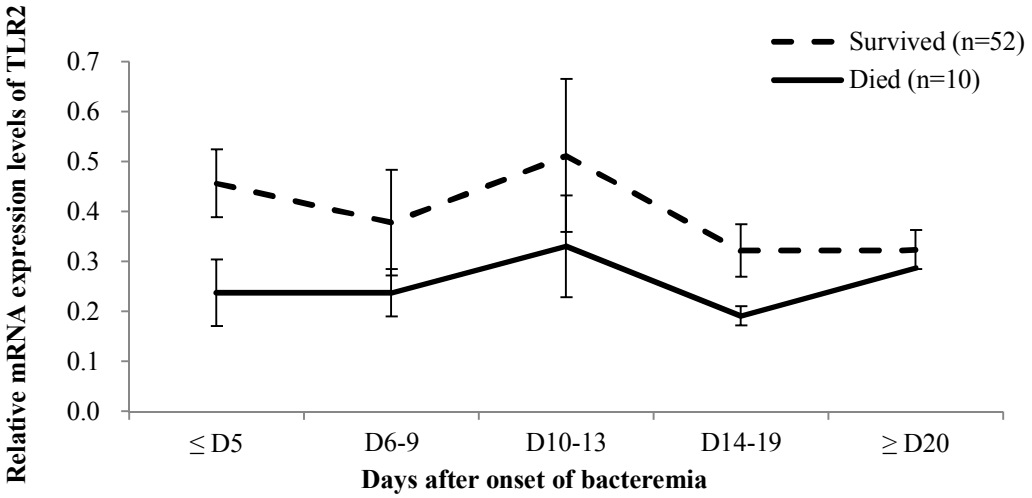


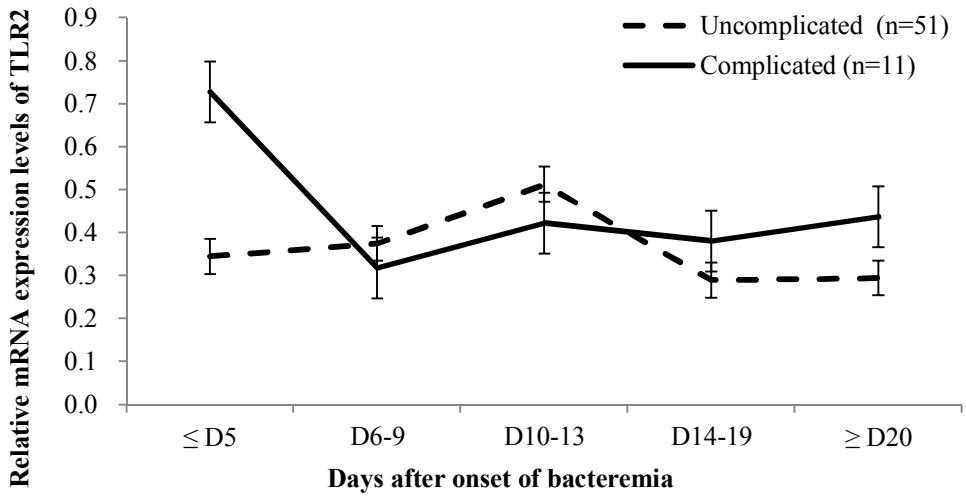
Figure 2. Relative mRNA expression levels of TLR2 relative to GAPDH in all patients.

Differential expression of TLR2 in patients with different clinical outcomes and features

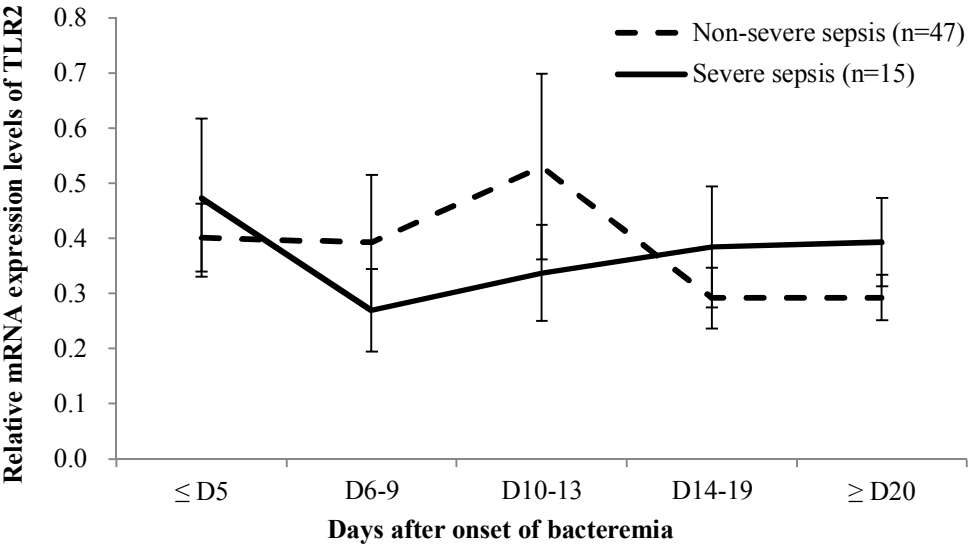
The changes in mRNA expression of TLR2 were compared between each clinical group (Figure 3). Since the samples were collected at different days of bacteremia in each patient, the time of sampling was divided into 5 periods as described in materials and methods. Those who died within 30 days of bacteremia (n=10) showed down-regulated TLR2 expression at ≤ D5 and less dynamic changes than those who survived. However, the level of TLR2 expression at ≤ D5 was higher in patients with complicated bacteremia than those with uncomplicated bacteremia (*P*=0.050). Patients with more severe infection such as septic shock and no early response also showed higher level of TLR2 expression at ≤ D5, although statistically not significant.



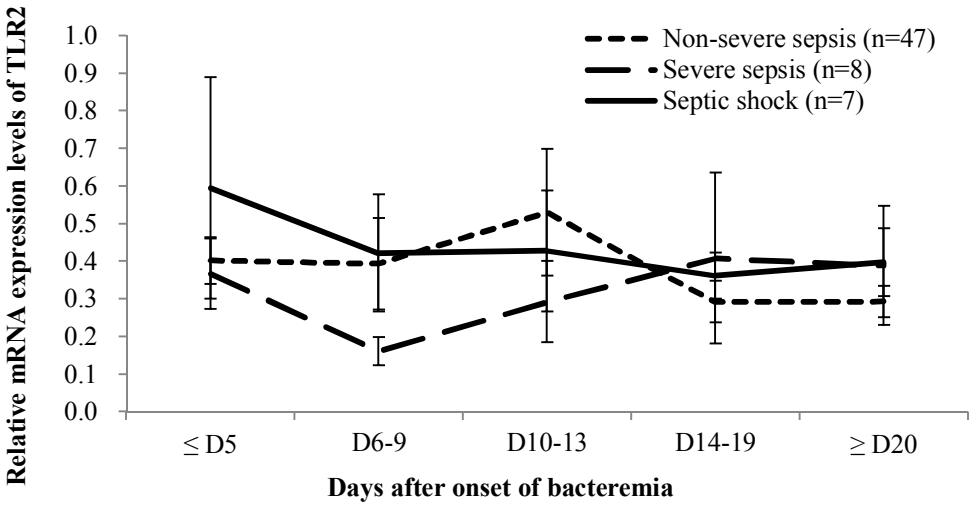
Survived	46	48	42	31	29
Died	9	5	5	2	1



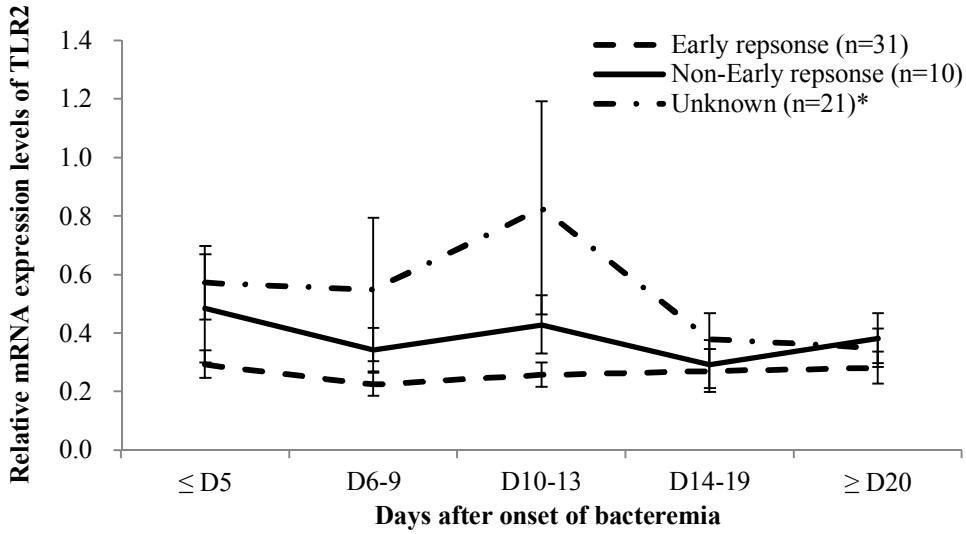
Uncomplicated	44	44	37	24	24
Complicated	11	9	10	9	6



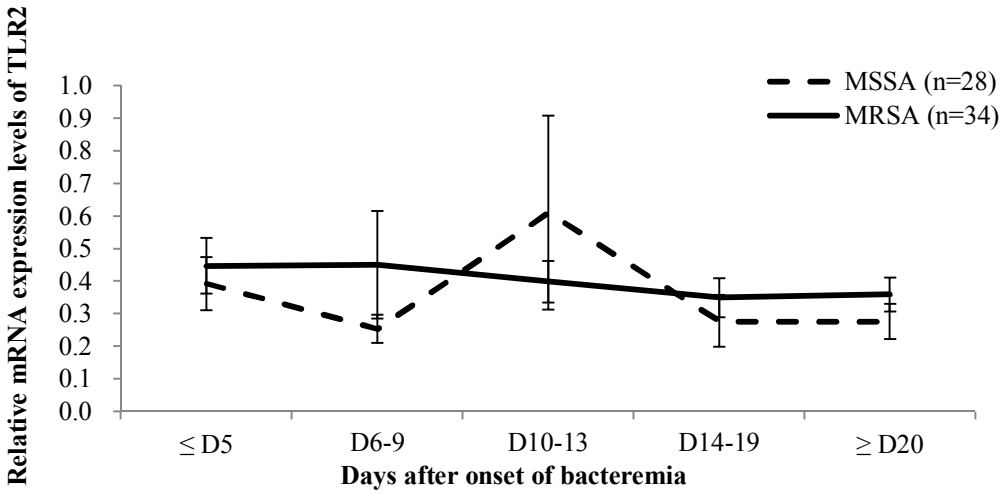
Non-severe sepsis	40	41	38	25	21
Severe sepsis	15	12	9	8	9



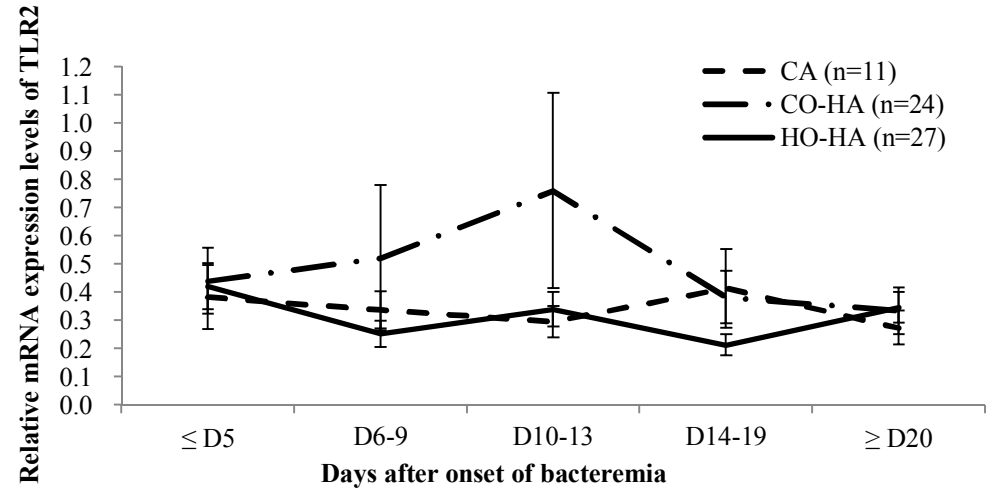
Non-severe sepsis	40	41	38	25	21
Severe sepsis	8	7	6	4	4
Septic shock	7	5	3	4	5



Early response	27	25	22	16	14
Non-early response	9	8	8	5	4
Unknown	19	20	17	12	12



MSSA	26	23	21	16	13
MRSA	29	30	26	17	17



CA	10	10	8	4	8
CO-HA	20	19	18	15	10
HO-HA	25	24	21	14	12

Figure 3. Relative mRNA expression levels of TLR2 in each clinical group. Information of number of patients in each time period is shown by the numbers under the graph. * Unknown culture result at 72 hours after appropriate treatment

mRNA expression levels of TLR2 in SAB patients compared to healthy controls

In 25 healthy volunteers, female was dominant (n=22, 88%) and the mean age was 32 years (range 22-46 years). The mean mRNA expression level of TLR2 was 0.255 ± 0.069 (range 0.143~0.399) (Figure 4).

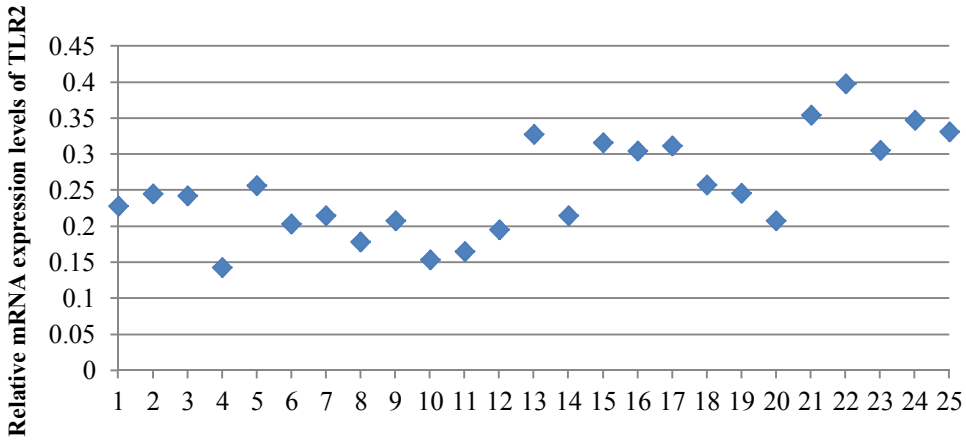


Figure 4. Relative mRNA expression levels of TLR2 in 25 healthy volunteers.

Only 1 or 2 samples were obtained in 14 patients. Therefore we compared the level of TLR2 expression for just first period, ≤ D5 of bacteremia between patients and healthy volunteers. Within 5 days of bacteremia, statistically significant difference was shown between the healthy volunteers and the survived group (P=0.006) (Figure 5). Mortality group showed lower level of TLR2 expression when compared to the survived group, although statistically not significant (P=0.172). TLR2 expression in patients with complicated

bacteremia was significantly increased compared with healthy volunteers (*P*=0.024) (Figure 6). The complicated bacteremia group showed higher TLR2 expression when compared to the uncomplicated bacteremia groups also, which was statistically not significant (*P*=0.050).

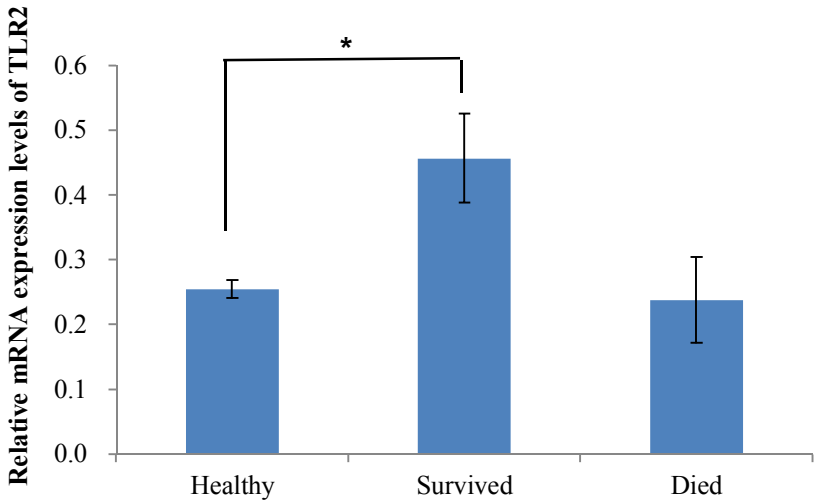


Figure 5. Relative mRNA expression levels of TLR2 in healthy volunteers (n=25), survived (n=46) and died (n=9) patients (within 5 days of bacteremia).

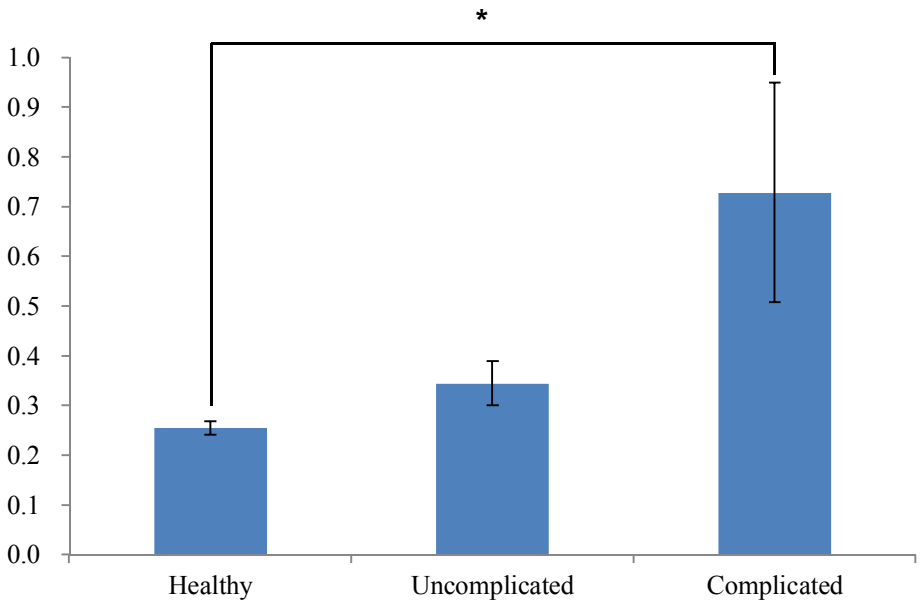


Figure 6. Relative mRNA expression levels of TLR2 in healthy volunteers (n=25), patients with uncomplicated bacteremia (n=42) and complicated bacteremia (n=13) (within 5 days of bacteremia).

Relationship between WBC with differential counts and level of TLR2 expression

Since TLR2 is expressed mainly in the hematopoietic cells, WBC with differential counts was analyzed to see the correlation with level of TLR2 expression. There was a weak positive correlation with proportion (%) of neutrophils (r=0.147, *P*=0.003) and absolute neutrophil counts (r=0.111, *P*=0.025) (Figure 7), and a negative correlation with proportion (%) of

lymphocytes (r=-0.137, *P*=0.006).

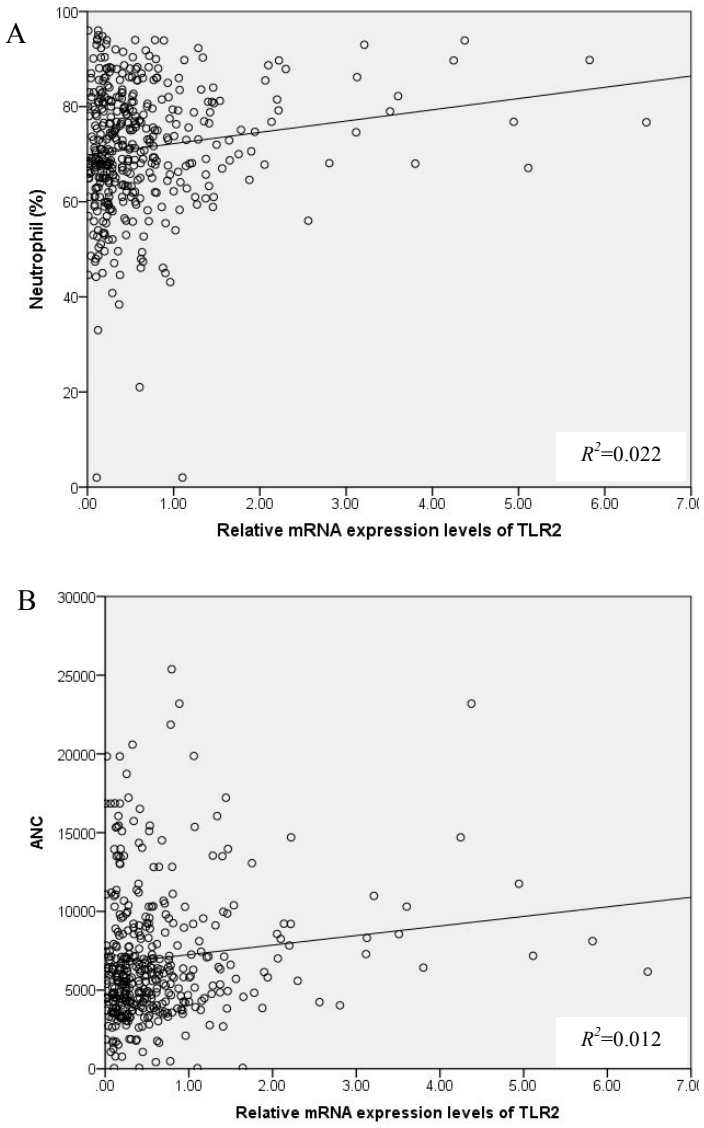


Figure 7. Correlation between relative mRNA expression level of TLR2 and A, Neutrophil proportion (%) in total WBC counts. B, Absolute neutrophil counts.

Association of cytokine concentrations with 30-day mortality

Proinflammatory cytokines, TNF-α and IL-6, and anti-inflammatory cytokine, IL-10, were measured from the earliest plasma obtained within a week of bacteremia occurrence (period 1) from 60 patients and between 7 and 14 days of bacteremia (period 2) from 54 patients. TNF-α was below the detection limit (<6.98 pg/mL) in all patients in both periods. IL-6 level above 10.60 pg/mL and IL-10 level above 2.49 pg/mL were measurable concentrations of the cytokines. In period 1, 87% (52/60) and 48% (29/60), and in period 2, 72% (39/54) and 41% (22/54) patients showed levels above the detection limit of IL-6 and IL-10, respectively. There was weak negative correlation between the day and the level of IL-6, implying that IL-6 tended to decrease as the day of bacteremia increased (P=0.044) (Figure 8). Those with 30-day mortality showed elevated IL-6 level in period 1 (Figure 9A) and period 2 (Figure 9B) which was statistically significant (P=0.005, P=0.006, respectively). IL-10 was also elevated in the mortal group compared with the survived group with more significant difference in period 1 than period 2 (P=0.001 and P=0.052, respectively) (Figure 10A and 10B). When the patients were stratified by unmeasurable (<2.49 pg/mL) or measurable (≥2.49 pg/mL) IL-10 concentrations, significantly more patients with 30-day mortality showed IL-10 ≥2.49 pg/mL in the first period (P=0.005) (Table 2). IL-6/IL-10 ratio was calculated and there was no significant difference between the mortal and the survived patients in both periods. However, in

period 1, the IL-6/IL-10 ratio tended to either high or low in the mortal patients whereas as it was distributed more evenly in the survival patients. The percentage of patients with IL-6/IL-10 ratio below 10 was 40% and 22%, and above 50 was 50% and 16% in the mortal and the survived patients, respectively, in period 1 (Figure 11).

There was no statistically significant correlation between the level of TLR2 expression and concentration of cytokines in plasma. TLR2 signaling triggers both proinflammatory and anti-inflammatory responses. In patients with 30-day mortality, TLR2 expression was down-regulated with more patients showing elevated IL6 and/or IL-10 level.

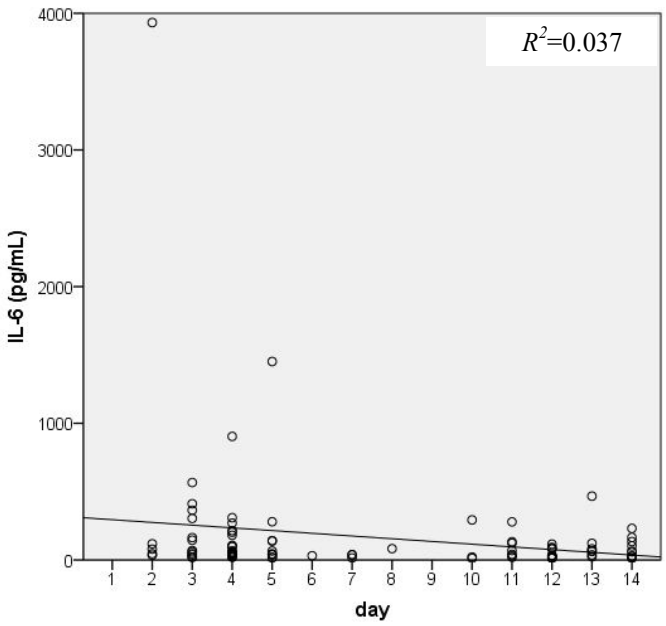


Figure 8. Changes in IL-6 level according to the day of bacteremia, measured from 91 samples of 55 patients.

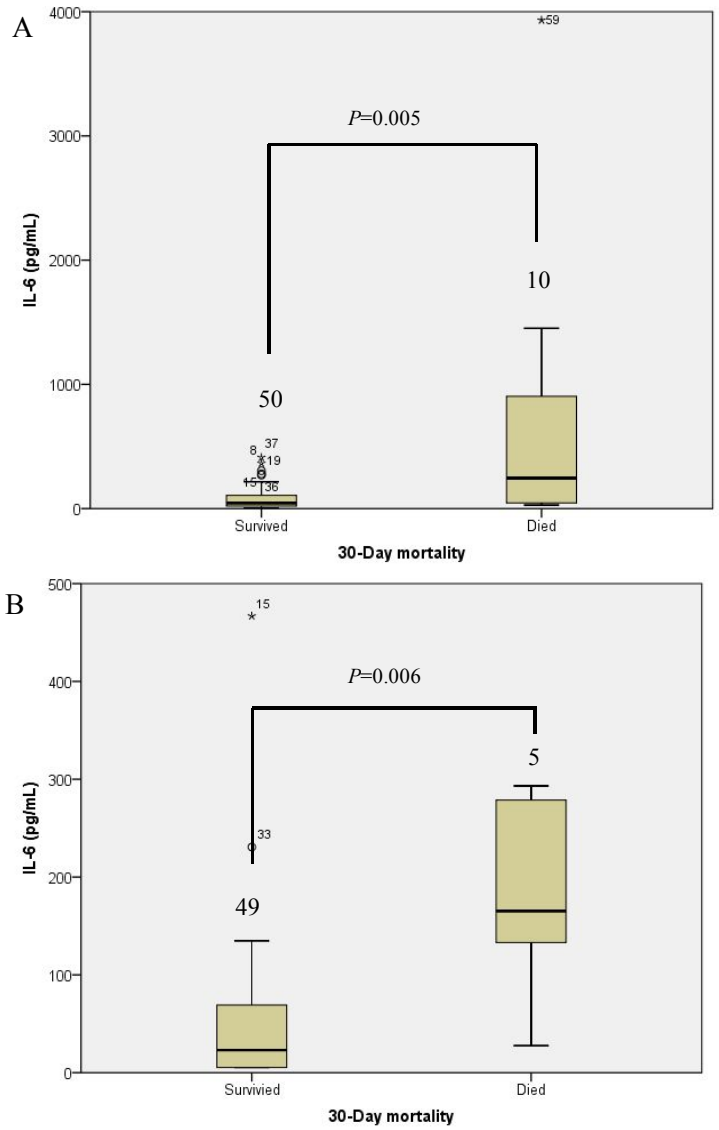


Figure 9. IL-6 level in bacteremia patients with survival and 30-day mortality. A, period 1 (n=60). B, period 2. (n=54)

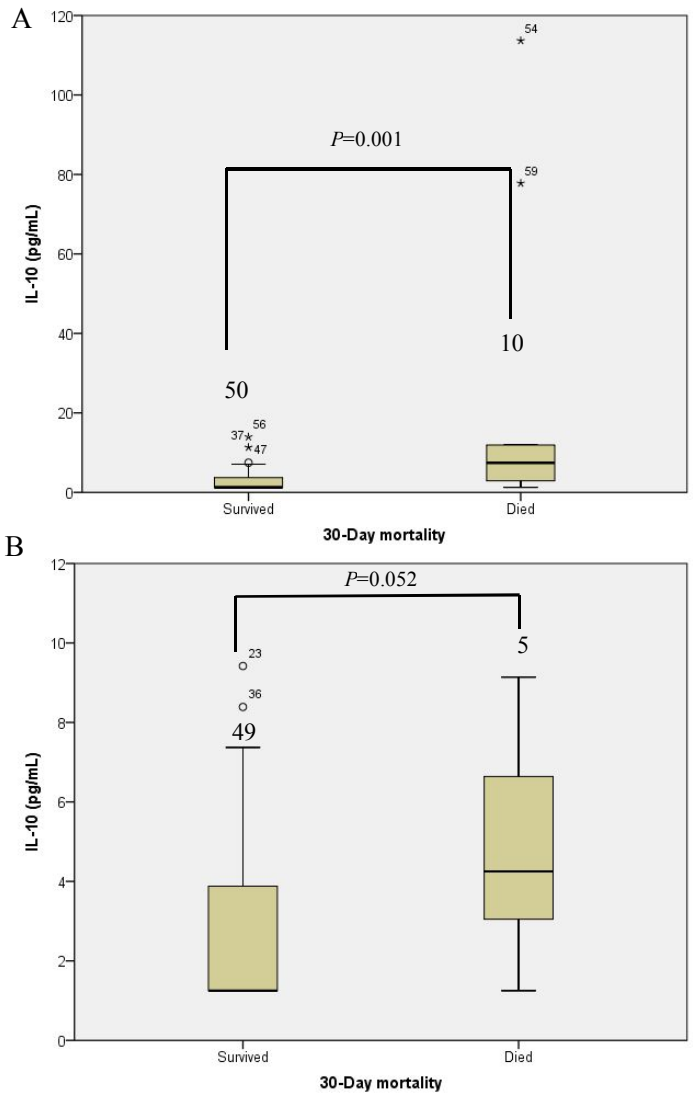


Figure 10. IL-10 level in bacteremia patients with survival and 30-day mortality. A, period 1 (n=60). B, period 2. (n=54)

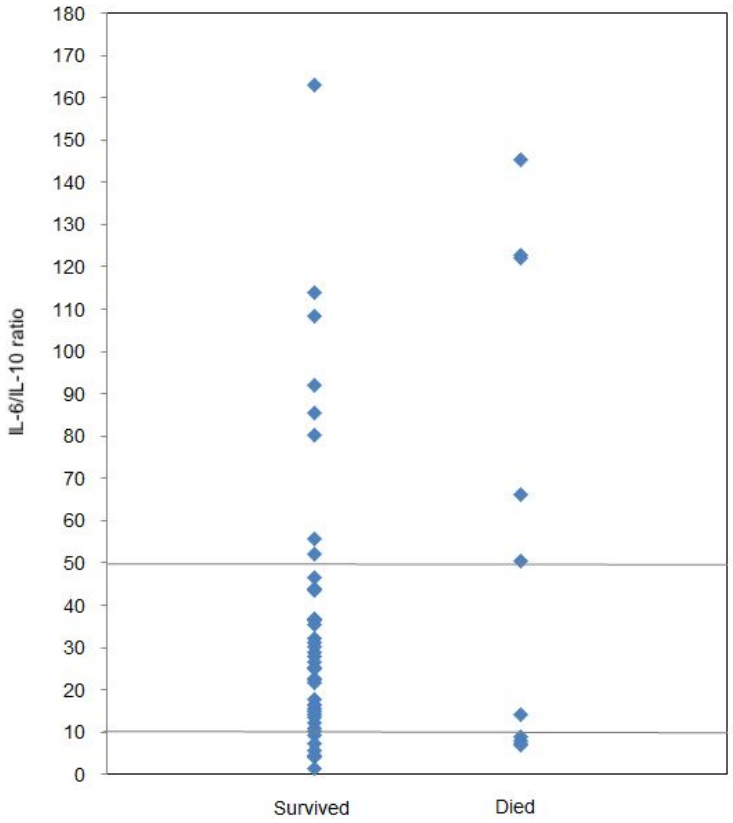


Figure 11. IL-6/IL-10 ratio of the patients with and without 30-day mortality in period 1.

Table 2. Cytokine Concentrations in the Patients with Survival and 30-day Mortality

Period 1	**Survival (n=50)**	**Death (n=10)**	*P* value[a]
IL-6			0.330
Not measurable (<10.60 pg/mL)	8 (16%)	0 (0%)	
Measurable (≥10.60pg/mL)	42 (84%)	10 (100%)	
IL-10			
Not measurable (<2.49 pg/mL)	30 (60%)	1 (10%)	0.005
Measurable (≥2.49 pg/mL)	20 (40%)	9 (90%)	
Period 2	**Survival (n=49)**	**Death (n=5)**	*P* value[a]
IL-6			0.306
Not measurable (<10.60 pg/mL)	15 (30.6%)	0 (0%)	
Measurable (≥10.60 pg/mL)	34 (69.4%)	5 (100%)	
IL-10			0.146
Not measurable (<2.49 pg/mL)	31 (63.3%)	1 (20%)	
Measurable (≥2.49 pg/mL)	18 (36.7%)	4 (80%)	

[a]Fisher exact test

Discussion

TLRs are known to play a key role in the innate immune response by inducing proinflammatory mediators (i.e. IL-1β, IL-6, TNF-α, etc.) and also anti-inflammatory mediators such as IL-10. Among the known TLRs, TLR2 is a key sensor for detecting *S. aureus* infection (35, 36). In the present study, mRNA expression of TLR2 was measured in SAB patients during the course of infection, in order to investigate the effect of TLR2 on clinical outcomes of 30-day mortality and complicated bacteremia. The mRNA expression levels of TLR2 during the course of bacteremia were variable among the patients. In the 30-day mortality group, the level of TLR2 expression was lower than the survival group, which is consistent with the previous study on sepsis patients (24). In general, the level of TLR2 expression at early stage (within 5 days of bacteremia) tended to be higher in patients with more severity such as septic shock, no early response and complicated bacteremia.

IL-6 is a proinflammatory cytokine which is elevated in acute phase of infection. In this study, in the group of patients with measurable IL-6 level, the increment was more prominent in patients with 30-day mortality compared to those who survived. Previous study showed significantly higher IL-6 levels in patients with complicated *S. aureus* bloodstream infection (37). In sepsis, elevated IL-6 correlated with severity (38) and death (39). IL-10 may represent immune response dysregulation or “immunoparalysis” via induction of apoptosis of antigen-presenting cells (40), and it was elevated in

mortal *SAB* patients in this study as shown in the previous study (41). Since there were more patients with measurable (≥2.49 pg/mL) IL-10 level in the mortality group at early stage, this may indicate immune response dysregulation in mortal group. In the recent study by Minejima et al, although IL-6 and IL-8 were found to be significantly elevated, IL-10 had the strongest association with the risk of persistent bacteremia and death (42). Moreover, the ratio of anti-inflammatory to proinflammatory cytokines (IL-10/TNF) at 72 hours was the strongest predictor of early response, similar to the previous study that showed significantly high IL-10/TNF ratio in nonsurvivors with severe sepsis (43).

Several studies showed increased mortality in TLR2-deficient mice compared to that in wild-type (WT) mice during *S. aureus* infection, which is thought to be due to TLR2 deficiency-induced defective phagocytosis, high bacterial burden, and the impairment of proinflammatory cytokine production (13, 44, 45). Another study gave similar findings of enhanced susceptibility to *S. aureus* infection and high bacterial burden in TLR2-deficient mouse, although excessive proinflammatory cytokine response was observed, in contrary (23). These findings suggest the importance of regulating proinflammatory and anti-inflammatory cytokine responses in protective TLR2 function during *S. aureus* infection. These two responses seem to be independent of each other (46).

TLR2 signaling pathway genes are differently regulated in PBMC and neutrophils of septic patients, and they are dynamically modulated in every

cell population throughout the different stages of sepsis (47). Although TLR2 and TLR4 receptors were significantly upregulated on both freshly isolated neutrophils and monocytes in patients of sepsis (48), the expression of TLR2 on neutrophils seems to be more dynamic than on monocytes (25). In this study we measured TLR2 expression on hematopoietic cells including both monocytes and neutrophils, and there was a positive correlation between TLR2 expression and proportion of neutrophil (%) and absolute neutrophil count. Considering that TLR2 expression of neutrophils may predominantly reflect the changes of immune response in bacterial infection, it would be worth measuring the TLR2 expression in neutrophils and monocytes separately.

Antibiotics such as vancomycin and linezolid are known to have effects on the innate immune response by upregulating TLRs including TLR2, and cytokines such as IL-6 (49). Since most of the patients (71.6%, 44/62) were using vancomycin for the infection treatment, there could be some effects on the expression of TLR2 and section of cytokines by use of antibiotics. There were 2 patients using both vancomycin and linezolid and 1 patient was using linezolid only. Antibiotics can alter inflammatory responses without antimicrobial activity, such as β-lactam antibiotics which trigger *mecA* expression and PBP2A in MRSA leading to high levels of secreted IL-1β and increased inflammation (50).

The host response in *S. aureus* infection is a complicated mechanism controlled by innate and adaptive immune immunity. TLR2, an innate

immune receptor, plays an important role in *S. aureus* infection either being protective or detrimental depending on the production and release of proinflammatory and immunomodulating cytokines. Many knock-out studies on mice as well as the autosomal recessive MyD88 and IRAK-4 patients demonstrated the importance of TLR2 and MyD88 in protection against *S. aureus* (51-54). Besides TLR2, additional host proteins are also likely to play key roles in host recognition of *S. aureus* (17). NOD2 (the nucleotide-binding oligomerization domain-containing protein 2) constitutes a primary intracellular microbial-recognition system that is parallel to, but independent of the extracellular TLR system (55, 56). The molecules involved in signaling pathway of TLR2 are also important factors that need to be considered.

Host immune response is one of the factors affecting the infection severity and prognosis. Other host factors such as age, gender, ethnicity, socioeconomic status, presence of comorbidities can influence mortality (57). Pathogen and environment are also the major factors involved. There are virulence factors of *S. aureus* such as Panton Valentine leukicidin (58), superantigen family (e.g. toxic shock syndrome toxin-1 and the staphylococcal enterotoxins), adhesion proteins like collagen adhesion protein (cna) which cause severe infections (59-63). Staphylococcal superantigen-like proteins (SSLs), a family of 14 proteins located on two genomic clusters is one of the secreted virulence factors (64-66). SSL3 was identified as a potent inhibitor of TLR2 (67, 68). SSL3 interferes with TLR2 activation at two stages: by blocking ligand binding and interacting with an already formed

TLR2-lipopeptide complex to prevent TLR heterodimerization and downstream signaling (2). There has been a heterogeneity of host TLR2 stimulation by *S. aureus* isolates as *S. aureus* isolates displayed considerable variability in TLR-activity with low to absent TLR2-activity in 64% of the isolates tested (68/106) (69). Research in a large cohort is necessary to fully understand the function of TLR2 in *S. aureus* infections, since it is hard to assess the effect of TLR2 expression with many other factors of the pathogen and the environment.

The discovery of Toll-like receptors and their ligands have opened the way to new therapeutic approaches (70). It is suggested that the inhibition of inflammasome signaling may turn out to be a useful adjuvant therapy in severe *S. aureus* infections (71). Furthermore, recombinant soluble TLR2 (sTLR2) acted as a physiological regulator of membrane-bound TLR2 signaling and resulted in modulation of the inflammatory response to live Gram-positive bacteria by competing with ligand-induced mobilization of TLR2 when administrated to mice (72). Conversely, upregulation of TLR2 with Pam3Cys, a synthetic ligand of TLR2, has shown a beneficial effect in the retinal innate immune response to *S. aureus* infection (14). A role for TLRs (specifically TLR2) and other PRRs is emerging in the crosstalk between the host and its microbiome (73). Therefore, understanding the in-vivo role of TLR2 in *S. aureus* infection through human studies like this study is needed to further develop theses novel therapeutic strategies.

There are a few limitations because this study was performed in real-life

routine clinical practice conditions. First, samples were not collected at the same time points, i.e. the day sample was drawn and the total number of samples from each patient was different. In order to analyze statistically, we arbitrarily assigned the sampling time point as ≤D5, D6-9, D10-13, D14-19 and ≥D20, which could have caused bias. Most of the first sample in each patient was collected at D3-5 which was quite comparable between the patients. Second, since we had to include only patients with informed consent, the number of patients with worse outcome was relatively small, because medically unstable patients were less likely to be enrolled. Third, we used samples that remained after the routine CBC tests, which had variable amount of blood in each patient. This resulted in different amount of RNA in each patient, and although we did our best to normalize, still some bias remains in the process of real-time PCR. However, we did our best to maintain the quality of the samples by extracting RNA within 6 hours of blood sampling and performing RT-PCR on the same day or at least within a month.

In conclusion, down-regulated TLR2 expression was associated with 30-day mortality in SAB. In the patients with 30-day mortality, either IL-6 or IL-10 was increased compared with those who survived, implying imbalance of proinflammatory and anti-inflammatory immune responses. This study suggests that TLR2 and cytokines may be considered as potential prognostic factors in *S. aureus* infection.

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국문 초록

서론: 황색포도알균은 흔한 원인 병원체로, 사람에 따라 무증상 집락화부터 중증 감염까지 광범위한 임상양상의 감염을 일으키고, 여기에 숙주 인자가 기여할 것으로 추정된다. 미생물의 pathogen associated molecular patterns(PAMPs)를 인지하는 pattern recognition receptors (PRRs) 역할을 하는 Toll like receptors (TLRs) 중 TLR2가 황색포도알균 감염에서 중요한 역할을 하는 것으로 알려져 있으며, 하위 신호전달 체계에서 사이토카인이 분비되어 감염증의 호전 혹은 악화에 기여할 것으로 추정하고 있다. 본 연구에서는 황색포도알균 균혈증 환자에서 TLR2의 발현과 사이토카인 분비가 예후와 연관성을 보이는지 평가하였다.

방법: 2014년 3월부터 2015년 4월까지 서울대학교 병원과 분당서울대학교 병원에서 황색포도알균 균혈증으로 진단된 환자를 대상으로 균혈증 발생 후 일정 시기(5일이내, 6-9일, 10-13일, 14-19일, 20일 이후)별로 혈액 검체를 수집하였다. EDTA 전혈을 원심분리 후 혈액세포에서 RNA를 추출하여 real-time PCR로 TLR2의 발현을 상대 정량 하였고, 혈장에서 Luminex를 이용하여 사이토카인을 측정하였다. TLR2 발현 정도 및 사이토카인과 균혈증 발생 30일 내 사망, 감염 중증도의 연관성을 살펴보았다.

결과: 총 290명의 황색포도알균 균혈증 환자 중 62명이 연구에 포함되었고, 균혈증 발생 후 2일에서 48일에 걸친 기간 동안 혈액 검체를 수집하였다. 환자마다 시간에 따른 TLR2 발현은 다양한 변화 양상을 보였다. 균혈증 발생 30일 내 사망한 10명의 환자에서는 생존군이나 대조군에 비하여 TLR2의 발현이 감소되어 있었고 시간에 따른 변화도 적었다. 또한 균혈증 발생 2주이내 측정한 IL-6와 IL-10이 증가되어 있었다. 균혈증 발생 7일 이내 IL-10이 사망군(90%, 9/10)에서 생존군 (40%, 20/50)에 비해 측정범위 이상(>2.49 pg/mL)인 경우가 많았다. 사망군에서 IL-6/IL-10 비율은 높거나 낮은 경향을 보였다. 중증 감염의 경우 균혈증 발생 5일이내 TLR2 발현이 상대적으로 증가되고 시간에 따른 변화가 더 역동적이어서 심한 면역 반응을 시사하였다.

결론: 황색포도알균 균혈증에서 30일 내 사망군에서 TLR2 발현이 감소하고 IL-6와 IL-10이 증가하였다. 중증 환자군에서는 면역 반응의 활성화로 초기 TLR2의 발현이 증가되었다. 이것은 TLR2 발현이나 사이토카인 분비와 같은 숙주 면역 반응이 황색포도알균 균혈증의 예후 예측 인자가 될 수 있는 가능성을 보여준다.

주요어: 황색포도알균, Toll-like receptor 2 (TLR2), 사이토카인

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